American Journal of Hematology 54:30–39 (1997)

Properties of the Mouse α-Globin HS-26: Relationship to HS-40, the Major Enhancer of Human α-Globin Gene Expression

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HS-26, the mouse homologue of HS-40, is the major regulatory element of the mouse α-globin gene locus. Like HS-40, HS-26 is located within an intron of a house-keeping gene; comparison of the nucleotide sequences of HS-26 and HS-40 reveals conservation of the sequences and positions of several DNA binding motifs in the 5′ regions of both elements (3 GATA, 2 NFE-2, and 1 CACCC sites) and the absence in HS-26 of three CACCC sites and one GATA site that are present in the 3′ region of HS-40, suggesting that the two elements might not be identical. We report here that when HS-26 is linked to a 1.5 kb PstI human α-globin gene fragment, it has a weak enhancer activity in induced MEL cells and in transgenic embryos, and it does not have any detectable activity in adult transgenic mice. This suggests that HS-26 does not have Locus Control Region (LCR) activity but can act as an enhancer during the embryonic life when integrated at a permissive locus. To further test the importance of HS-26 at its natural locus, we have generated embryonic stem cells and chimeric animals in which 350 bp containing HS-26 have been replaced by a neomycin resistance gene by homologous recombination. The sizes of the chimeras' red cells were then estimated by measuring forward scattering on a FacsScan apparatus in hypotonic conditions. This revealed that a fraction of the chimeric animals' red cells were smaller than normal mouse red cells and were very similar to cells from mice heterozygous for α-thalassemia. Density gradient analysis also suggested the presence of thalassemic cells. These results indicated that despite its lack of LCR activity, HS-26 is important for the regulation of the mouse α-globin gene locus. Am. J. Hematol. 54:30–39, 1997

Key words: α-globin; hyper-sensitive site; enhancer; transcription

INTRODUCTION

The organization of the α-globin gene locus in human and mouse is similar: nucleotide sequence homologies between the two species extends for at least 100 kb upstream from the α-globin genes [1] and the same housekeeping genes are located downstream from the globin genes [2] (Fig. 1). Expression studies in MEL cells and transgenic animals have shown that HS-40, a DNase I hypersensitive site located 40 Kb upstream from the human ζ-globin gene, is the major regulator of the human locus since it has a strong enhancer activity on the human α-globin gene while no other fragment of the locus has any detectable activity [1,3–5].

In the mouse locus, sequencing and chromatin studies have revealed the presence of HS-26, a DNase I hypersensitive site located 26 Kb upstream from the ζ-gene. As in the human locus, HS-26 is located within the intron of a house keeping-gene [1]. Interestingly, detailed sequence comparisons reveal that the 5′ region of both elements contain almost identical GATA, CACC, and NFE-2 bind-

Contract grant sponsor: NIH; Contract grant number: HL38655; Contract grant sponsor: AHA; Contract grant number: 95015110.

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Received 9 May 1996; Accepted 7 August 1996

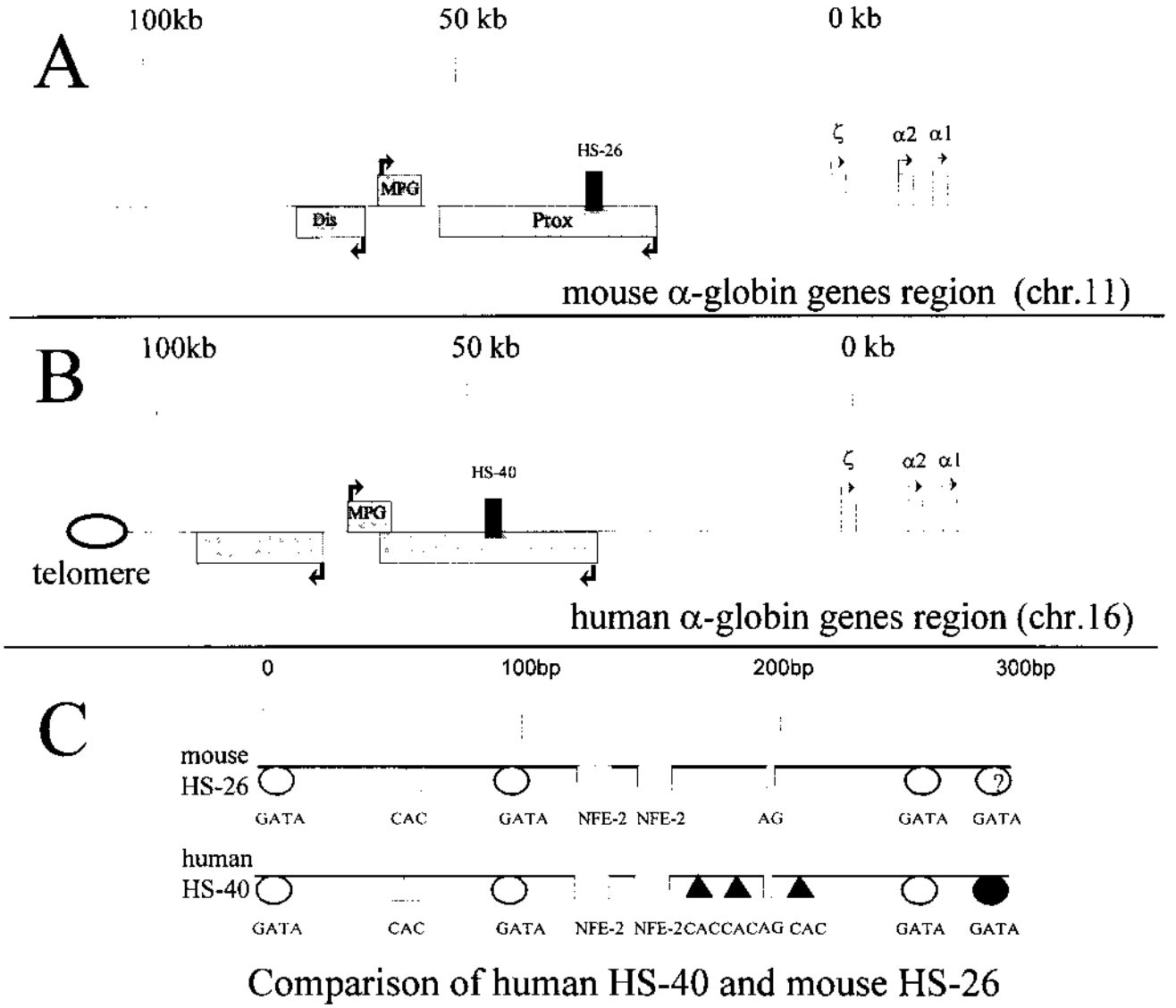


Fig. 1. The human and mouse α-globin gene loci. A and B: Large scale map of the human and mouse α-globin gene cluster. The ζ gene is an α-like globin gene expressed during embryonic life. The α1 and α2 genes are expressed throughout development and are the only genes expressed during adult life. The Prox, MPG, and Dis genes are house-keeping genes located near the α-globin gene cluster in both the human and murin species. HS26 and HS40 (represented by black rectangles) are located inside an intron of the Prox gene. For clarity, the pseudo-genes and the τ gene that are present at these loci are not represented. C: Detailed map of HS-26 and HS-40: comparison of the trans-acting factor binding sites of the core sequences of HS-40 and HS-26. Schematic representation of a 300 bp DNA segment containing HS-40 and HS-26. GATA, CAC, NFE-2, and AG boxes are binding sites for transcription factors that are probably important for the function of HS-26 and HS-40. The binding sites represented by open boxes are perfectly conserved between the human and murin enhancers. The binding sites represented by closed boxes are absent from the mouse version. The position and orientation of the binding sites located in the 5′ portions of both elements are perfectly conserved. In contrast, the 3′ parts of HS-26 and HS-40 are very different (three CAC and one GATA sites missing). The question mark on the most 3′ GATA site denotes a binding site that differs by one point mutation between HS-26 and HS-40.

ing sites while their 3′ regions are quite divergent since three CACC sites and one GATA site are present in the human but not in the mouse version [6]. Foot-printing and band-shift studies have demonstrated that many but not all of the binding sites described above bind their cognate factors in vitro [7].

We report here that HS-26 is a much weaker enhancer than HS-40 in stably tranfected MEL cells and in

transgenic mice when linked to the human α-globin gene. This suggests that the CACC and GATA sites present in HS-40 but not in HS-26 are important for enhancer activity and that additional regulatory elements present somewhere in the mouse locus are needed for full expression. In addition, we have deleted a 350 bp HS-26 fragment by homologous recombination in ES cells and assessed the resulting phenotype in chimeric animals. The red cells of the chimeric animals appeared thalassemic, suggesting that HS-26 is important for the regulation of the locus.

MATERIALS AND METHODS

Cell Cultures

MEL cells were grown at 37°C in 5% CO_2 in DMEM supplemented with 10% fetal calf serum and 1 × Penn/Strep. MEL cells differentiation was achieved by incubation for 4.5 days in the same medium supplemented by 5 mM HMBA. This resulted in levels of differentiation >90% as determined by benzidine staining.

ES cells were grown on a Mitomycin C inactivated mouse fibroblast feeder cells layer in 7.5% CO_2 at 37°C in DME supplemented with 10% FCS, LIF, β-mercaptoethanol, non-essential amino acids, glutamine, and nucleosides.

Plasmids

The plasmids used in these studies were constructed using standard methods and are depicted in Figure 2A to C.

Transfection

Electroporations of MEL cells were performed as described by Moon and Ley [8] using 5.10^6 cells and 10 μg of gel purified insert DNA. Pools of 25 clones and individual clones were isolated.

Copy Number Determination

Ten micrograms of genomic DNA extracted from MEL cells or transgenic mice tails, were digested with HincII and XcmI and hybridized with a 350 base fragment containing HS-26 (Fig. 2). This resulted in a 1.3 and 1.7 kb internal fragment for the transgene and a 2.7 and 2.2 kb for the endogenous fragment. Estimates of the copy number were then derived by comparison of both bands on a phosphor-imager.

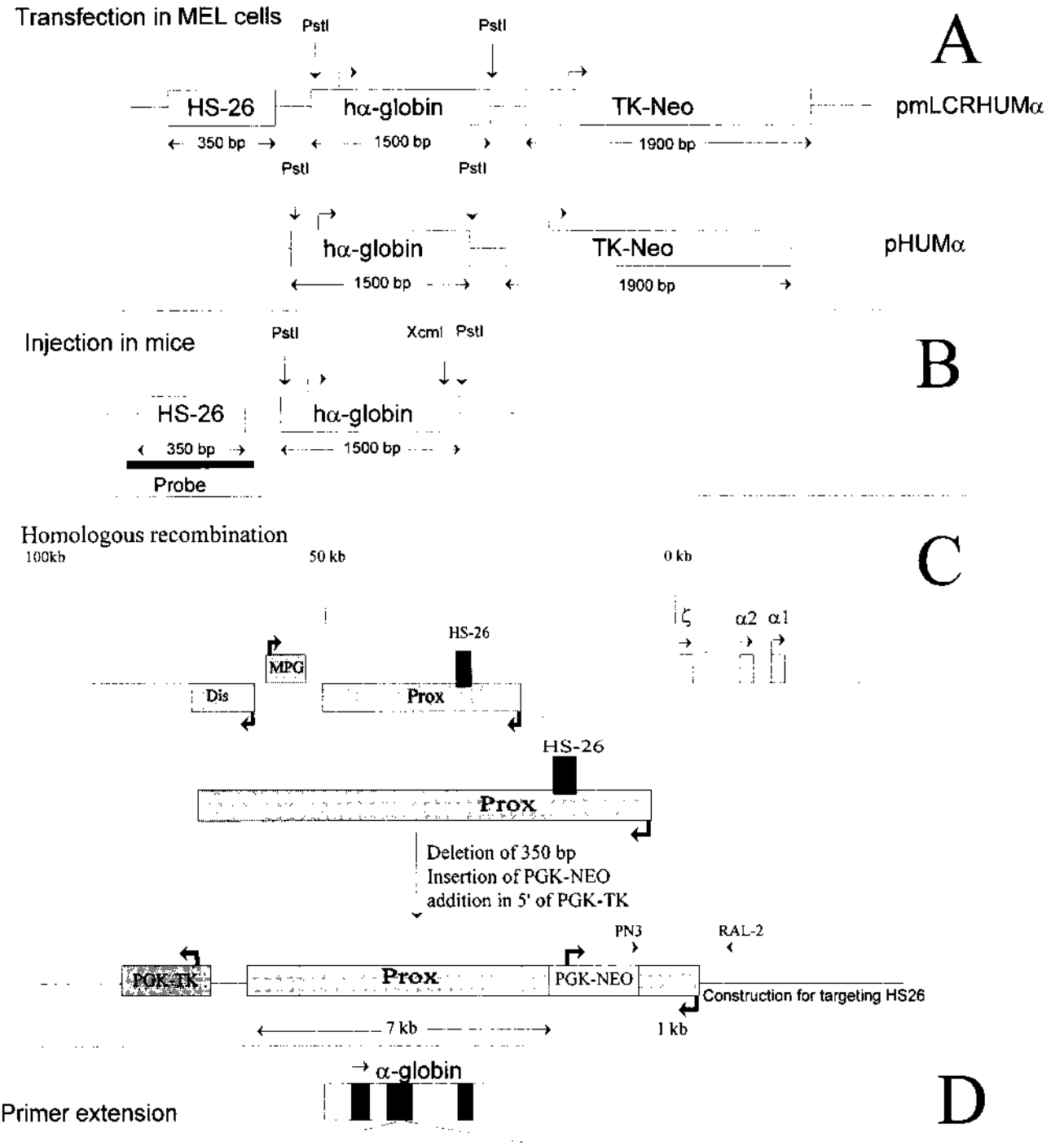
ES Cells

Identification of homologous recombinant ES cells was performed by Southern blots after digestion with EcoRI, SacI, BamHI, HindIII, XbaI, or KpnI. With all these enzymes, differences between the endogenous and the knockout chromosomes were as predicted by the map (data not shown). These results were confirmed by PCR analysis using primer PN3 (in the PGK-NEO gene) and RAL3 (outside of the construct) (Fig. 2).

RNA Quantification

RNA was extracted from uninduced and induced MEL cells using a guanidium thiocyanate based procedure (RNAzol B kit, TEL-TEST, Friendswood, Texas). Reticulocyte RNAs were extracted from peripheral blood using the procedure of Nienhuis et al. [9]. Embryonic red cells were collected for each embryo and frozen in liquid N_2. Once the identification of the transgenic embryos had been performed by Southern blot, red cells from

Fig. 2. Plasmid constructs used during this study. A: Fragment analyzed in MEL cells. The two DNA fragments used in the MEL cells transfection studies are depicted. pmLCRHUMα contains HS-26 linked to the human α-globin gene and to the TK-Neo gene (the gene that we used to select the transfected cells). pHUMα is identical to pMLCRHUMα except that it does not contain HS-26. Comparison of the human α-globin gene expression levels obtained with these two plasmids revealed that HS-26 is a weak enhancer in induced MEL cells. B: Transgenic studies. The DNA fragment that was injected in fertilized eggs to obtain transgenic animal is depicted. The fragment is identical to pmLCRHumα except for the absence of the selectable marker. The probe and the restriction sites used for copy number determination in transgenic mice and in transfected MEL cells are depicted in the third line. C: Homologous recombination studies. The construct used to replace HS-26 by the PGK-Neo gene in ES cells is depicted. The first two lines represent the mouse α-globin locus endogenous locus. The last line represents the construct that used to target HS-26: a 350 bp fragment containing HS-26 was deleted and replaced with the PGK-Neo gene (a positive selection marker). In addition the PGK-TK gene (a negative selection marker to enrich in homologous recombinant) was ligated 5′ to the left arm of our targeting construct. PN3 is one of the primers that was used for PCR identification of the Neo^R and Gan^R ES cells subclones that had integrated our construct by homologous recombination and, therefore, contained a chromosome 11 in which HS-26 was deleted and replaced by the PGK-Neo gene. The second primer is not shown but was located just 3′ of the 1 kb fragment flanking the PGK-Neo gene. D: Quantification of the mouse and human α-globin RNA. Human and mouse α-globin mRNAs were quantified in MEL cells and in transgenic animals by primer extension using primer P1, a primer that anneals with nucleotides 13 to 42 of exon 2 of both the human and mouse globin mRNA. The human and murin mRNA are identical in this region except for two mismatches at positions 33 and 36; to insure proper annealing with both mRNA, primer P1 was synthesized with an Inosine at these two positions. The primer extensions were carried out in the absence of dTTP and in the presence of ddTTP. This results in the synthesis of a 35 bp cDNA when P1 annealed with the murin α-globin mRNA and in the synthesis of a 44 bp cDNA when P1 annealed with the human mRNA. THe cDNAs were then separated by denaturing poly-acrylamide gel electrophoreses and quantified by phosphorimaging.



GAT GTT TGC TAG CTT CCC CAC CAC CAA GAC CTA CTT CCC TCA CTT mouse α-globin

GG GTG GTG GTT CTG GAT GAA IGG IGT GAA Primer P1

ddT

GAT GTT CTT GTC CTT CCC CAC CAC CAA GAC CTA CTT CCC GCA CTT human α-globin

GG GTG GTG GTT CTG GAT GAA IGG IGT GAA Primer P1

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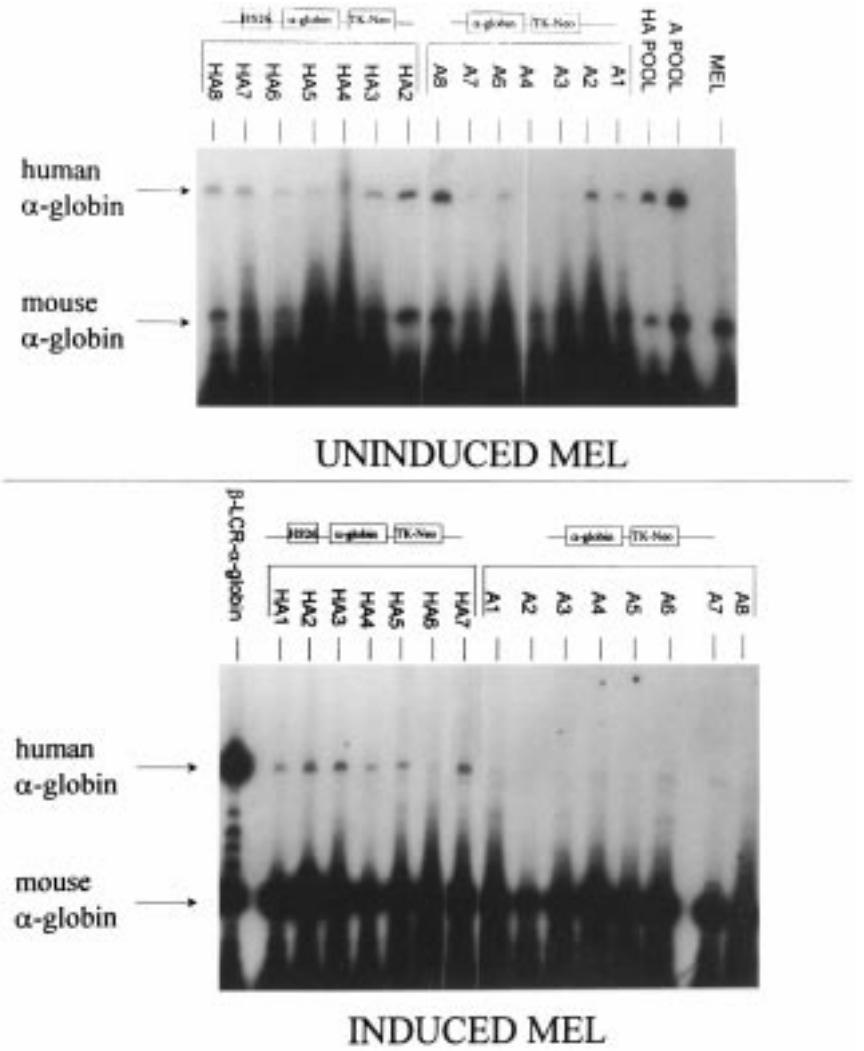

Fig. 3. Primer extension analysis of MEL cells transfected with the human α-globin gene. One hundred micrograms of total RNA extracted from uninduced MEL cells and 30 μg of RNA extracted from induced MEL cells were used as templates in primer extension reactions with 32P labeled primer P1. The cDNAs were separated by 8M urea poly-acrylamide gel electrophoresis and 48 hr exposure autoradiographies were performed on the dried gels. Quantification was performed after 1 to 24 hr exposure in a phosphor-imager cassette. HA1 to HA8: individual sub-clones of MEL cells transfected with the pmLCRHUMα construct. A1 to A8: individual sub-clones of MEL cells transfected with the pHUMα construct. A pool and HA pools: pools of 25–50 colonies. The copy number in all the single colonies was two, one, or less than 1. In uninduced MEL cells, HS-26 appears to have no effect. In induced MEL cells, HS-26 has a small but reproducible enhancer activity. All the experiments were performed in duplicate.

transgenic embryos from the same litter were pooled and their RNA extracted.

Primer extensions were performed as described by Sambrook et al. [10] using primer P1, a primer that is complementary to both the mouse and human α-globin cDNA (Fig. 2). The reactions were performed both in the absence of dTTP and the presence of ddTTP. This resulted in the production of a 35 base fragment for the mouse gene and a 44 base fragment for the human gene (Fig. 2). Gel purified 32P 5′-labelled primer P1 (100,000 cpm) was annealed with 100 μg of uninduced MEL cells RNA, 30 μg of induced MEL cells RNA, 3 μg of adult reticulocyte RNA, or 1 μg of embryonic reticulocyte RNA. The cDNAs were separated on a 15% 8M urea polyacrylamide gel and were quantified by a Molecular Dynamic (Sunnyvale, CA) phosphor-imager.

Chromatographic Separation of the Globin Chains

HPLCs were performed as previously described [11] using a three-steps acetonitrile gradient ranging from 35 to 55%.

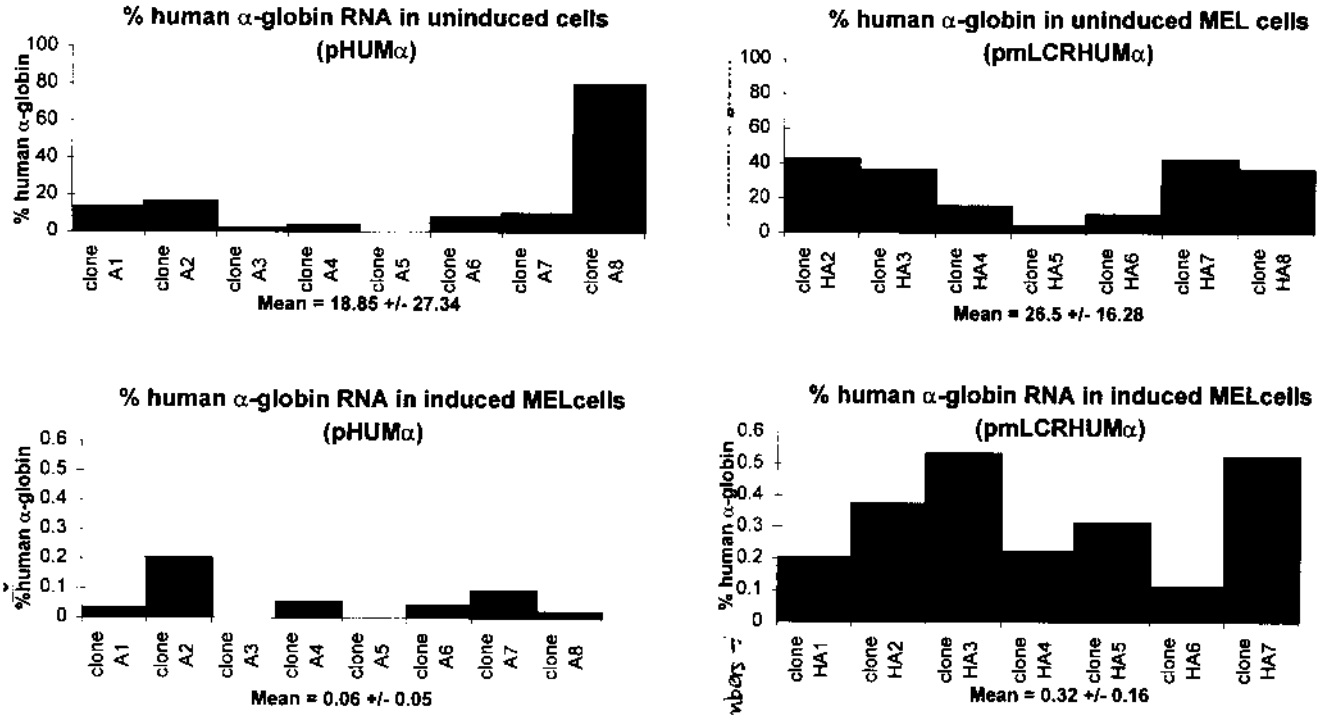

Fig. 4. HS-26 is a weak enhancer in induced MEL cells. Total RNA from induced and uninduced MEL cell sub-clones containing the hα-globin gene alone (clone A1 to A8) or linked to HS-26 (clone HA1 to HA) were extracted and the levels of α-globin RNA were determined by primer extension and quantified by phosphor-imaging. The levels of human α-globin RNA are expressed as % of the levels of mouse α-globin mRNA. Since there are four mouse α-globin genes and on average only one integrated copy of the human α-globin gene, the % presented in these graphs should be multiplied by four to obtained the level of human α-globin gene per copy of mouse α-globin gene. In uninduced MEL cells, the human α-globin mRNA averaged 18.6 ± 27.34% of the mouse α-globin mRNA in the absence of HS-26 and 26.5 ± 16.28 in the presence of HS-26, a difference that is not statistically significant ($P = 0.6$). In induced MEL cells the human α-globin mRNA averaged 0.06 ± 0.005% of the mouse α-globin mRNA in the absence of HS-26 and 0.32 ± 0.16% in the presence of HS-26, a difference that is statistically significant ($P = 0.0016$). We have, therefore, concluded that HS-26 has no activity in uninduced MEL cells and has a weak enhancer activity in induced MEL cells.

Percoll-Larex (Stractan) Density Gradients

Percoll-Larex (Stractan) density gradients were performed as previously described [11].

Flow Cytometry

Separations of normal and thalassemic red cells were performed as described by Van den Bos et al. [12]. The red cells were washed twice in PBS, resuspended in 103 mM NaCl and the Forward Light Scatter was measured on an Applied Biosystem (Foster City, CA) FACSCAN flow cytometer.

RESULTS

Analysis of HS-26 in MEL Cells

Two plasmids containing a 1.6 kb PstI human α-globin gene fragment linked to the TK-Neo gene with (pHS-26humα) or without (pHumα) HS-26 linked in 5′ (Fig. 2A) were transfected by electroporation in MEL cells and pools of 25 to 50 G418 resistant clones and 8 individual colonies were then isolated. The number of copies of integrated constructs were then assessed by Southern blots and the expression levels of the human α-globin gene (α^H) in induced and uninduced cells were compared to the expression of the mouse endogenous α-globin gene (α^M) by primer extension as described in Materials and Methods.

Copy-number analysis revealed that the individual clones had integrated two, one or less than one copy of the transgenes. The results of the primer extension analysis are illustrated in Figure 3 and summarized in Figure 4. In uninduced MEL cells, the expression of α^H ranged from 2 to 80% (mean = 18.8 ± 27.34%) of α^M in the absence of HS-26 and from 4 to 42% (mean 26.5 ± 16.28%) of α^M in the presence of HS-26. In induced cells, expression of α^H in all clones was very low (mean = 0.06 ± 0.05%) in the absence of HS-26 and ranged from 0.1 to 0.5% of α^M (mean = 0.32 ± 0.05%) in the presence of HS-26. Statistical analysis (*t*-test) revealed that the differences observed in the presence or absence

of HS-26 were not significant in uninduced MEL cells but were highly significant in induced MEL cells ($P = 0.0016$).

Analysis of HS-26 in Transgenic Animals

A fragment containing the human α-globin gene linked to HS-26 was isolated from plasmid pHS-26humα (Fig. 2B) and was injected into the male pronucleus of fertilized mouse eggs. This resulted in the production of five transgenic founders. Southern blot analysis revealed the presence of 3, 4, 6, 7, and 13 copies of the transgenes. HPLC analysis showed that none of the adult founders expressed any detectable amount of human globin gene in their peripheral blood (Fig. 5). Primer extension analysis of reticulocyte RNA revealed traces of α^H RNA in four of the five founders (Fig. 5). Analysis of 12 day embryos from three of the founders revealed that in one of the three lines (copy number = 13) the α^H gene was expressed at about 3% of the α^M gene (about 1% on a per copy basis) on both the protein and RNA level (Fig. 5).

Knocking-Out HS-26

A plasmid containing the PGK-TK gene linked to a 6 kb genomic fragment in which the 350 bp core HS-26 fragment was deleted and replaced by the PGK-NEO gene was constructed (Fig. 2C). After electroporation and positive-negative selection, fifty G418 and Gancyclovir resistant clones were isolated and screened by Southern blot and PCR analysis for the presence of a disrupted HS-26 region. One of the fifty clones had undergone insertion of the transgene by homologous recombination of the α-globin gene cluster and, therefore, contained the PGKNEO gene in place of the 350 by HS-26 fragment. Chimeric animals were generated by blastocyst injection of the recombinant ES cells. Four chimeric animals with ES cell contributions ranging from 5–10% (as judged by the coat colors) and 8 animals with smaller ES contributions were obtained and further analyzed by density gradient and flow cytometry.

Density gradient revealed that some of the chimeras had a large amount of low density red cells indicating that they might be thalassemic (Fig. 6). Determinations of the mean corpuscular volume and the mean hemoglobin concentration with a Coulter (Haleah, FL) Counter were compatible with this interpretation. Two of the chimeras were then further analyzed by flow cytometry: red cells rendered spherical by incubation in a hypotonic medium were analyzed by measuring forward light scatter (FLS) in a FACSCAN apparatus. Comparison of the FLS pattern in normal, β-thalassemic mice and mice chimeric for cells heterozygous for the deletion of HS-26 clearly revealed the presence of abnormally sized and shaped cells in the chimeras.

DISCUSSION

The cis-acting elements regulating the human α-globin genes have been extensively characterized; comparisons to the human β-globin gene cluster have underlined important similarities and differences between the regulation of the two gene clusters (reviewed in [13]). It is well established that HS-40 is an essential regulatory element of the α-globin gene cluster and that some of the transcription factors that bind HS-40 also bind the β-globin Locus Control Region (LCR) and promoter. However, while the β-globin LCR can confer high-level position-independent, copy number dependent expression on linked transgenes [14,15], the expression driven by HS-40 is not related to the number of integrated-copies [4]. In addition, by contrast with observations made on the β-globin cluster [16], absence of HS-40 did not produce any detectable change in chromatin structure or replication timing of the α-globin cluster [7]. In this study, we have begun to analyze the function of the mouse α-globin gene cluster cis-regulatory sequences, using transfection in MEL cells, transgenic mice, and homologous recombination technique in ES cells that should allow us to precisely dissect the locus in ways that are not possible with the human homologue.

Our results in uninduced MEL cells have shown that on a per copy basis α^H was expressed on average at close to 100% of the level or the mouse endogenous locus in the absence of HS-26, therefore, suggesting that HS-26 is not required at this stage of differentiation.

As had been previously reported [17], in induced MEL cells and in the absence of HS-40 the human α-globin gene is expressed at very low levels and might actually be down-regulated when compared to the uninduced clones. Presence of HS-26 had a small but clearly detectable enhancer effect. The relatively large range of expression observed in the single-copy clones and the low levels of expression (about 2%/copy of mouse α-globin) indicated that the expression of the human α-globin gene was influenced by the site of integration and, therefore, that HS-26 alone cannot be regarded as an LCR such as the β-globin LCR.

In transgenic animals, only traces of human α-globin mRNA could be observed in the adult stage and only one of the three transgenic lines analyzed displayed a small level of expression in the embryonic stage. These results confirm and extend the results of Gourdon et al. [7] who have shown that transgenic mouse embryos express very variable levels of human α-globin mRNA [7]. Previous studies have shown that in the absence of a β-globin LCR, the human α-globin gene was totally silent in both the embryonic and adult stages of development [18,19]. Our results demonstrate that HS-26 has a small enhancer effect in the embryonic stage of expression but that ex-

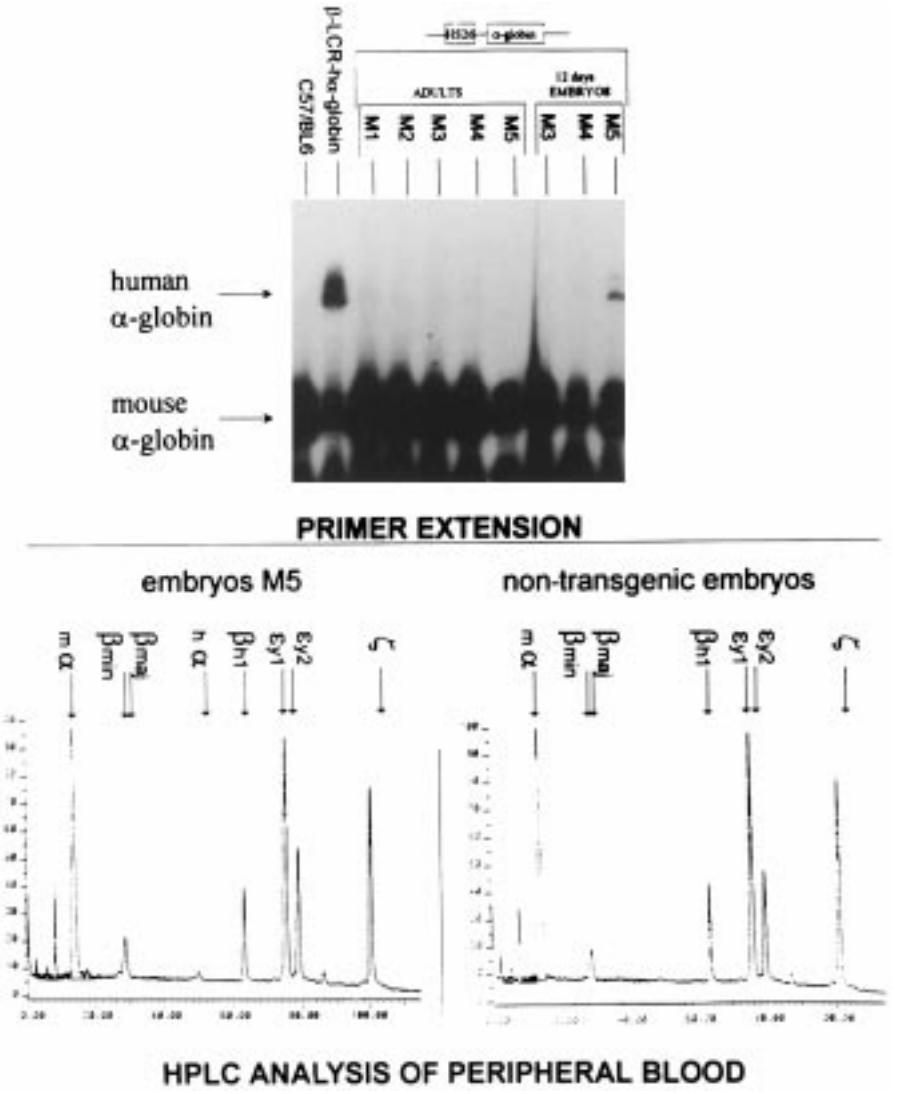

Fig. 5. Primer extension and HPLC analysis of mice transgenic for the HS-26α-globin construct. Primer extension: Reticulocyte RNAs were extracted from whole blood by the procedure of Nienhuis et al. [9] and analyzed by primer extension as described in the legend of Figure 3. C57/BL6 = non-transgenic mice (negative control). β-LCR-hα-globin = mouse transgenic for the human α-globin gene linked to the β-globin LCR (positive control); M1 to M5: Five transgenic adult founders that all express trace amounts of human α-globin; M3 to M5 (embryos) = pools of 12 day transgenic embryos derived from adult mice M3 to M5. M5 expresses low levels of adult globin. HPLC analysis: globin chains from embryos M5 and from a mixture of non-transgenic embryos were separated by HPLC as described in Materials and Methods. Embryo M5 expresses low levels of human α-globin chains. No α-globin chains were detected in embryo from the M3 and M4 lines (data not shown).

pression probably depends on the presence of other regulatory elements provided by the integration sites.

The enhancer activity of HS-26 that we observed in MEL cells was clearly much smaller than what has been reported for the human HS-40 [5]. Similarly, the lack of enhancer activity of HS-26 on a linked human α-globin gene in adult mice clearly contrasts with the strong enhancer activity of HS-40 in transgenic mice that has been previously reported [4,8]. Since the 5′ regions of HS-26 and HS-40 are strikingly similar, these differences are probably caused by the absence of CACC and GATA binding sites in the 3′ region of HS-26. Differences between HS-26 and HS-40 could be explained by the hypothesis that the absence of the CACC and GATA sites in the mouse version are compensated for by changes in the architecture of the promoters. The low enhancer activity observed in our experiment could then be due to a partial incompatibility of mouse HS-26 with the human α-globin promoter. Alternatively, the absence of the CACC and GATA sites in HS-26 might be compensated for by the presence of other regulatory elements in the mouse locus. Recently, we have identified four erythroid

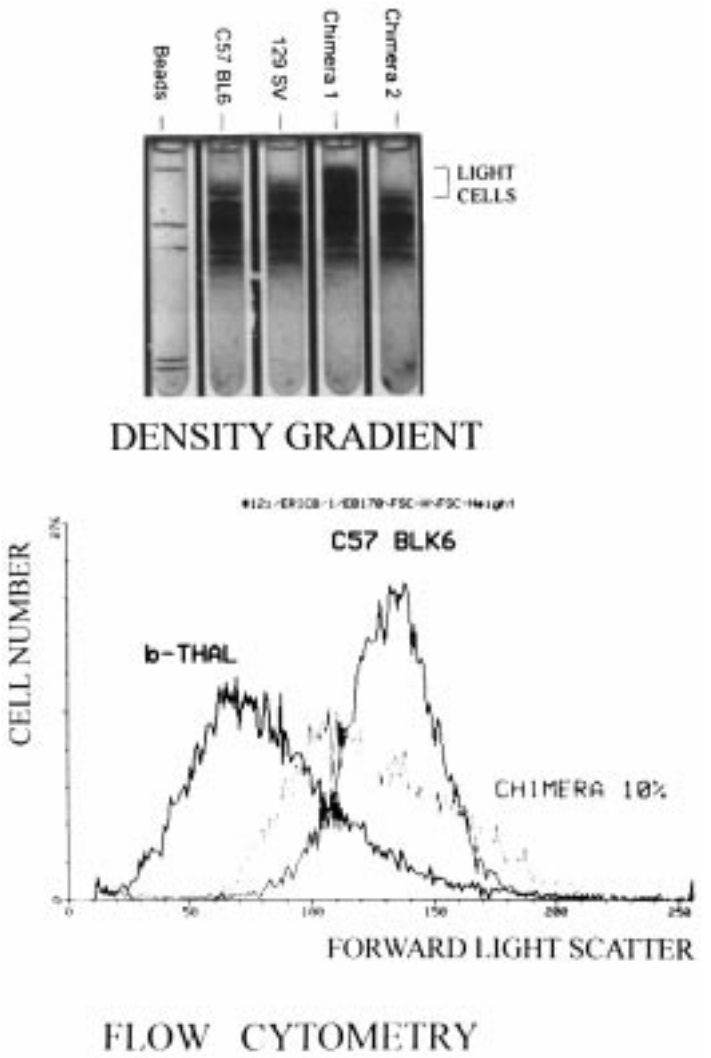

Fig. 6. Analysis of red blood cells from mouse chimeric for the HS-26 knock-out. Percoll-Larex density gradient: red blood cells were separated according to density by the method of [11]. C57/BL6 and 129/Sv = red blood cells from strain C57/BL6 and 129/Sv. Chimera 1 and 2: chimeric mice composed of 90–95% normal C57/BL6 cells and 5–10% knock-out 129/Sv cells (% determined by the coat color). Chimera 1 exhibited an increase in the number of light cells in three different experiments. Flow cytometry: Forward light scatter pattern from red blood cell rendered spherical by resuspension in an hypotonic buffer. β-thal: red blood cells from mice heterozygous for a β^{maj} knock-out. C57/BL6: RBC from normal C57/BL6 mice. Chimera 10%. RBC from chimera 1 (see above). The RBC from the β-thalassemic mouse have a FLS pattern shifted to the left probably because of their smaller size. RBC from chimera 1 are also shifted to the right but to a smaller extent and have a much broader size distribution, suggesting that a proportion of the cells are thalassemic. RBC from mice heterozygous for α-thalassemia were also analyzed and were also shifted to the left (data not shown).

specific DNaseI hyper-sensitive sites upstream from the mouse α-globin gene clusters [20] that could act in concert with HS-26. A third hypothesis that cannot be eliminated is that one or several mouse trans-acting factors are incompatible with the human promoter. This possibly could explain the inhibition of human α-globin expression upon induction of MEL cells. Further experiments will be required to discriminate between these possibilities.

The hypothesis that HS-26 is the mouse homologue of HS-40 is based on the fact that this region of the mouse α-globin gene locus is hypersensitive to DNAse I and on the sequence homologies with the human counterpart that were subsequently observed [1,6]. To determine the importance of HS-26 for the in vivo regulation of the mouse α-globin gene cluster, we have replaced a 350 bp fragment containing HS-26 by the PGK-Neo gene in embryonic stem cells and have produced chimeric animals. Analysis of these animals' red blood cells (RBC) by two independent methods clearly demonstrated that presence of a subpopulation of RBC that is less dense and smaller than

normal mouse RBC. This could be interpreted by the hypothesis that HS-26 is important for the activation of the mouse globin genes, but one cannot exclude that the presence of abnormal RBC in the chimera was due to the presence of the PGK-NEO gene rather than to the HS-26 deletion, since it has previously been shown that presence of a selection marker between the LCR and the globin gene could profoundly disrupt the expression of the β-globin gene [21,22]. Nevertheless, similar experiments performed on HS-40 by Bernet et al. [22] suggest strongly that the presence of the NEO gene in this type of construct does not affect the expression of the α-globin gene.

We conclude that HS-26 is a weak enhancer in MEl cells as well as during the embryonic stage of development, that in itself it does not have the properties of the β-LCR but that it is nevertheless important for the regulation of the locus.

ACKNOWLEDGMENTS

This work was supported by NIH grant HL38655 and AHA grant 95015110.

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